

Role of altered renal lipid metabolism and the sterol regulatory element binding proteins in the pathogenesis of age-related renal disease

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Role of altered renal lipid metabolism and the sterol regulatory element binding proteins in the pathogenesis of age-related renal disease.

Background. There are well-known changes in age-related renal function and structure, including glomerulosclerosis and decline in glomerular filtration rate (GFR). The purpose of this study was to identify a potential role for lipids in mediating age-related renal disease.

Methods. Mice of five different age groups (3, 6, 12, 19, and 23 months old) were studied.

Results. We have found that in C57BL/6 mice there was a progressive increase in age-related glomerulosclerosis [increase in periodic acid-Schiff (PAS) staining and accumulation of extracellular matrix proteins including type IV collagen and fibronectin], increased glomerular basement thickness and podocyte width and effacement, and increased proteinuria. These changes were associated with age-related increase in lipid accumulation as determined by increased Oil Red O staining in kidney sections. Biochemical analysis indicated that these lipid deposits corresponded to significant increases in renal triglyceride and cholesterol content. We have also found significant age-related increases in the nuclear transcription factors, sterol regulatory element-binding proteins (SREBP-1 and SREBP-2), protein abundance and increased expression or activity of their target enzymes that play an important role in lipid synthesis.

Conclusion. Our results indicated that there was an age-related increase in renal expression of SREBP-1 and SREBP-2 with resultant increases in lipid synthesis and triglyceride and cholesterol accumulation in the kidney. Because we have previously shown that increased expression of SREBPs in the kidney per se results in glomerulosclerosis and proteinuria, our data suggested that increased SREBPs' expression resulting in increased renal lipid accumulation may play an important role in age-related nephropathy.

Key words: SREBPs, cholesterol, triglycerides, nephropathy, glomerular basement membrane thickness, podocyte width.

Received for publication August 5, 2004

and in revised form June 12, 2005

Accepted for publication July 14, 2005

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The normal aging process in humans and in experimental animals is associated with decreases in renal blood flow (RBF), glomerular filtration rate (GFR), glomerulosclerosis, and alterations in tubular function, including functionally important defects in concentration, dilution, acidification, and phosphate transport. Recent data from the Third National Health and Nutrition Examination Survey (NHANES III) indicate that in addition to diabetes and hypertension, age is also a major predictor of chronic kidney disease (CKD), which is characterized by decline in kidney function (GFR less than 60 mL/min/1.73 m²) and presence of kidney damage (albuminuria). The incidence of CKD increases as a function of age and 15% of individuals older than 65 years without concomitant hypertension or diabetes have CKD [1].

During disease states the decreases in RBF and GFR become of major clinical significance as they interfere with normal drug excretion and predispose the elderly to increased incidence of ischemic and toxic acute renal failure [2]. In addition, the age-related glomerulosclerosis and tubulointerstitial fibrosis predispose the elderly to a progressive decline in renal function caused by diabetes mellitus, hypertension, and other systemic diseases associated with glomerulosclerosis. Furthermore, presence of CKD results in major complications such as anemia, bone disease, and malnutrition, and CKD is definitely a major independent risk factor for cardiovascular disease and all-cause mortality [3].

The elderly are at least five times more prone to develop end-stage renal disease (ESRD) than young adults [1]. Both European and United States registries highlight the growing incidence of elderly subjects initiating dialysis [4, 5]. Increasing prevalence of renal disease in a growing elderly population necessitates understanding the anatomic, physiologic, and pathologic mechanisms involved in the aging kidney.

Age-related glomerulosclerosis and tubulointerstitial fibrosis with loss of GFR may not necessarily be irreversible consequences of aging. Interestingly, longitudinal follow up of 254 healthy elderly subjects over 23 years old with repeated creatinine clearances revealed that one third of the subjects had no absolute age-related decrease in creatinine clearance [6]. Similarly, studies performed mainly in rodents suggested that glomerulosclerosis and tubulointerstitial fibrosis are not necessarily inevitable consequences of the aging process, that several factors, including angiotensin II [7], advanced glycosylation end products [8], oxidative stress [9], and nitric oxide [10], may play a role in age-related renal disease and that modulation of these processes may help prevent or at least attenuate the age-related increase in glomerulosclerosis and decline in renal function.

Several of the above metabolic and cellular processes that modulate age-related glomerulosclerosis are remarkably similar to metabolic and cellular processes that play an important role in the pathogenesis and progression of kidney disease in diabetes mellitus [11–14]. In recent studies we found that in a model of type 1 diabetes in the rat there was increased renal accumulation of lipids which was mediated by increased expression of the sterol regulatory element-binding protein-1 (SREBP-1). In cultured mesangial cells high glucose medium also stimulated increased expression of SREBP-1 and in SREBP-1a transgenic mice, in the absence of any increases in serum glucose or serum lipids, there was increased accumulation of triglyceride and cholesterol in the kidney which was associated with increased expression of transforming growth factor- β (TGF- β) and vascular endothelial growth factor (VEGF), and the extracellular matrix proteins type IV collagen and fibronectin, resulting in glomerulosclerosis and proteinuria [15]. This study indicated that increased expression of SREBP-1 plays an important role in the pathogenesis of diabetic kidney disease.

The SREBP has been described as a master regulator of both fatty acid and cholesterol metabolism [16, 17]. Three SREBP isoforms have been identified and characterized, SREBP-1a, SREBP-1c, and SREBP-2. Studies in transgenic mice overexpressing each of the three SREBP isoforms in the liver have indicated that SREBP-1c plays a preferential role in fatty acid synthesis compared to cholesterol synthesis, whereas SREBP-2 plays a preferential role in cholesterol synthesis compared to fatty acid synthesis [16, 17].

In view of the similarities between mediators of age-related and diabetes-induced glomerulosclerosis, we performed the present study to determine if age-related glomerulosclerosis was associated with increased renal expression of SREBPs with resultant increased accumulation of lipids in the kidney.

METHODS

Animal models

We obtained 3-month-old, 6-month-old, 12-month-old, 19-month-old, and 23-month-old male C57BL/6 mice (18 in each group) from the NIA aging colony, and housed them at the animal facility at the Denver Veterans Affairs Medical System. The mice were maintained on a 12-hour light/dark cycle and fed standard rodent chow (Purina rodent chow 5015) (Ralston Purina Co., St. Louis, MO, USA) ad libitum.

Spot urine samples were obtained on all mice. After the urine collection, 12 mice in each age group were sacrificed by intraperitoneal injection of pentobarbital. One kidney was freeze-clamped in liquid nitrogen for subsequent (1) RNA extraction and quantitative real-time polymerase chain reaction (PCR), (2) measurement of enzyme activity, (3) measurement of lipid composition, and (4) Western blotting. The other kidney was homogenized as described below for isolation of nuclei.

In order to eliminate the confounding variable of different dietary status between animals on some of the measured variables, one group of mice ($N = 6$) were sacrificed after a 48-hour fasting and another group of mice ($N = 6$) after a 48-hour fasting followed by 4 hours of refeeding.

In addition, six mice in each age group underwent in vivo perfusion fixation and the kidneys were then processed for histologic stains, immunofluorescence microscopy, and electron microscopy as described below.

Blood and urine chemistries

Serum glucose was analyzed using the glucose C2 Kit (Wako Chemicals USA, Inc., Richmond, VA, USA) [18]. Serum cholesterol was analyzed using the cholesterol CII Kit (Wako Chemicals USA, Inc.) [19]. Serum triglycerides were determined by the L Type TG H Kit (Wako Chemicals USA, Inc.) [20]. Serum free fatty acids were determined using the NEFA C Kit (Wako Chemicals USA, Inc.) [21]. Serum creatinine concentration was determined by the Jaffe's reaction of alkaline picrate with creatinine via the Creatinine Companion Kit (Exocell, Philadelphia, PA, USA) [22]. Blood urea nitrogen (BUN) was analyzed using the L-Type UN Kit (Wako Chemicals USA, Inc.) based on enzymatic assay utilizing urease and glutamate dehydrogenase. Urine albumin concentration was determined by competitive enzyme-linked immunosorbent assay (ELISA) via the Albuwell M Kit (Exocell) per the manufacturer's protocol. Urine creatinine was analyzed using the same kit as serum creatinine. Results were expressed as the urine albumin to creatinine ratio (mg/g).

Homogenate and nuclei isolation

Kidneys were homogenized at 4°C in homogenization buffer [20 mmol/L Tris-HCl, pH 7.4, 75 mmol/L NaCl, 2 mmol/L ethyleneglycol tetraacetate (EGTA), 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L Na₃VO₄, and 1 mmol/L dithiothreitol (DTT)], supplemented with a protease inhibitor cocktail consisting of 104 mmol/L AEBSF, 0.08 mmol/L aprotinin, 2 mmol/L leupeptin, 4 mmol/L bestatin, 1.5 mmol/L pepstatin A, and 1.4 mmol/L E-64 (Sigma-Aldrich, St. Louis, MO, USA).

Nuclear extracts were prepared according to the method of Morooka et al [23] with minor modifications. An aliquot of the homogenate was centrifuged at 3300 × g for 15 minutes at 4°C. The supernatants were discarded and the packed nuclear volume was estimated. The nuclei were resuspended in 0.5 packed nuclear volume of low-salt buffer [20 mmol/L Hepes, pH 7.9, 25% glycerol, 1.5 mmol/L MgCl₂, 20 mmol/L KCl, 0.2 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.5 mmol/L DTT]. A 0.5 packed nuclear volume of high-salt buffer (20 mmol/L Hepes, pH 7.9, 25% glycerol, 1.5 mmol/L MgCl₂, 0.8 mol/L KCl, 0.2 mmol/L EDTA, 0.2 mmol/L PMSF, and 0.5 mmol/L DTT) was then added. After 30 minutes of continuous gentle mixing, the nuclei were pelleted by centrifugation (30 minutes at 25,000 × g). The supernatants were saved and the protein concentration was determined by the method of Lowry et al [24]. The nuclear extracts were stored at -80°C.

Protein electrophoresis and Western blotting

Nuclear extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% wt/vol), as described by Laemmli [25] and then transferred to nitrocellulose membranes. Membranes were blocked in 5% dried milk in TBST [0.2% Tween-20 in 1× Tris-buffered saline (TBS)], incubated with (1) anti-SREBP-1 or (2) anti-SREBP-2 (1:1000) (BD Biosciences, Pharmingen, San Jose, CA, USA) antibodies followed by horseradish peroxidase-labeled anti-mouse IgG (1:5000 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), washed four times with 1× TBS, and then developed using the chemiluminescence detection system (Pierce Biotechnology, Rockford, IL, USA). The signals were quantified in a Phosphor Imager with chemiluminescence detector and densitometry software (Bio-Rad Laboratories, Hercules, CA, USA). Kidney homogenates were similarly subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were then probed with anti-3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (1:500) (Upstate Biotechnology, Lake Placid, NY, USA) antibody followed by horseradish peroxidase-labeled an-

tirabbit IgG (1:5000 dilution; Molecular Probes, Inc., Eugene, OR, USA) and processed as above.

Lipid extraction and analysis

Total lipid was extracted from kidney using the method of Bligh and Dyer [26]. Total cholesterol and triglycerides were determined using enzyme assays as described above.

Enzyme activity assay

Adenosine triphosphate (ATP) citrate lyase activity [27] was determined as described by Corrigan and Rider [28]. The assay solution, in a final volume of 1.00 mL, consisted of 50 mmol/L Tris-HCl, pH 7.8, 10 mmol/L MgCl₂, 100 mmol/L KCl, 5 mmol/L ATP, 0.2 mmol/L coenzyme A, 20 mmol/L potassium citrate, 0.15 mmol/L nicotinamide adenine dinucleotide (NADH), 10 mmol/L DTT, 2 U malate dehydrogenase, and kidney homogenate sample. Assays were preincubated for 7 minutes at 37°C and the reaction was started by the addition of coenzyme A. The decrease in absorbance at 340 nm was measured in a Varian-Cary 100 Bio UV-Vis Spectrophotometer (Varian, Palo Alto, CA, USA).

RNA extraction and quantitative real-time PCR

Total RNA was isolated from kidney using TRIzol (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using reverse transcript reagents (Bio-Rad Laboratories). The mRNA level was quantified using Bio-Rad iCycler Real Time PCR system. Briefly, 25 µL total reaction mix contained 20 ng of reverse-transcribed cDNA, 100 nmol/L forward/reverse primers and 1× iQ Supermix (Bio-Rad Laboratories). Cyclophilin was used as internal control and the amount of RNA was calculated by the comparative C_T method as recommended by the manufacturer. All the data were calculated from triplicate reactions. The sequences used were indicated in Table 1.

Perfusion fixation of mouse kidney

Mice were anesthetized and perfused with a fixative buffer that consisted of 3% paraformaldehyde and 0.05% picric acid in a 6:4 mixture of cacodylate buffer (pH 7.4, adjusted to 300 mosmol with sucrose) and 10% hydroxyethyl starch. After 5 minutes of fixation, the mice were perfused for an additional 5 minutes with the cacodylate buffer [15].

Periodic acid-Schiff (PAS) and Oil Red O staining and immunofluorescence microscopy

Paraffin sections were stained for hematoxylin and eosin and PAS. Frozen sections were used for Oil Red

Table 1. Primers for real-time polymerase chain reaction (PCR)

	Forward	Reverse
Cyclophilin	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT
SREBP-1c	GGCACTAAGTGCCTCAACCT	GCCACATAGATCTCTGCCAGTGT
ACO	GGCCAACTATGGTGGACATCA	ACCAATCTGGCTGCACGAA
CPT-1	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
HMG-CoA reductase	AGCCGAAGCAGCACATGAT	CTTGTTGAATGCCTTGTGATTG
ABCA-1	CGTTTCCGGGAAGTGTCTTA	GCTAGAGATGACAAGGAGGATGGA
SR-B1	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA
LDL receptor	GAAGTCGACACTGTACTGACCACC	CTCCTCATTCCCTCTGAAAGCCAT

Abbreviations are: ACO, acetyl coenzyme A oxidase; CPT-1, carnitine palmitoyltransferase I; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ABCA1, ATP-binding cassette A-1; SR-B1, scavenger-receptor class B, type 1; LDL receptor, low-density lipoprotein receptor.

O staining to determine the renal accumulation of neutral fats. The stained kidney sections were imaged with an Olympus microscope and scored semiquantitatively in a blinded manner by the renal pathologist (M.S.L.).

For immunofluorescence microscopy, frozen sections were preincubated for 10 minutes at room temperature with 3% milk powder in phosphate-buffered saline (PBS) containing 0.05% Triton X-100. They were then covered overnight at 4°C with the type IV collagen or fibronectin antibody (Chemicon, Temecula, CA, USA) diluted 1:200 in the preincubation solution. The sections were rinsed three times with PBS before incubation for 1 hour at 4°C with the secondary antibody, goat antirabbit IgG conjugated to Alexa 488 (Molecular Probes, Inc.). After rinsing with PBS, the sections were mounted using Dako-Glycergel™ (Dakopatts, Glostrup, Denmark) plus 2.5% 1,4-diazabicyclo-[2.2.2]-octane (DABCO) (Sigma-Aldrich) as a fading retardant. The kidney sections were then imaged with a laser scanning confocal microscope (Zeiss LSM 510; Zeiss, Thornwood, NY, USA).

Electron microscopy

Tissue was fixed in 3% paraformaldehyde in a 6:4 mixture of cacodylate buffer (pH 7.4, adjusted to 300 mosmol with sucrose) and 10% hydroxyethyl starch. The tissue was then postfixed in 1% buffered osmium tetroxide. The sample was dehydrated in a graded series of ethanol and embedded in an epoxy resin. Tissue was surveyed with a series of 1 µm sections for a representative sample. The selected specimens were thin sectioned, viewed, and photographed with a Phillips electron microscope 201 (Phillips Electron Optics, Inc., Mahwah, NJ, USA). The sections were read by the renal pathologist (M.S.L.) in a blinded fashion for determination of basement membrane thickness and podocyte morphology.

Statistical analysis

The results were expressed as mean ± SEM. The statistical significance of differences was assessed by one-way analysis of variance (ANOVA) with post hoc analysis with Student-Newman-Keuls test for multiple comparisons.

RESULTS

Body weights, kidney weights, and serum chemistries

Table 2 showed the body weights, kidney weights, and kidney weight/body weight ratio of the mice. As expected, the older mice weighed more and had larger kidneys. There was no significant difference in the kidney weight to body weight ratio. Table 3 showed serum values for glucose, triglycerides, cholesterol, and free fatty acids for the mice of various ages. Although there were significant changes in fasting versus postprandial serum glucose, triglycerides, cholesterol, and free fatty acids, there were no significant age-related changes in the serum values. Table 4 showed serum values for creatinine and BUN. There were no significant age-related changes in them.

Aging was associated with increases in glomerular PAS staining, glomerular basement membrane thickness, increased podocyte width and effacement, increased accumulation of extracellular matrix proteins, and proteinuria

An age-related increase in PAS staining was observed, which was most evident in mice 12 months and older (Fig. 1). Electron microscopy of the same kidneys showed that there was an age-related increase in glomerular basement thickness (Figs. 2 and 3A), and podocyte width (Figs. 2 and 3B). Podocyte effacement was also more prominent in the oldest animals, with some 23-month-old mice showing up to 80% effacement (Fig. 2). Immunofluorescence microscopy revealed that the increase in PAS staining was associated with age-related increases in type IV collagen (Fig. 4) and fibronectin (Fig. 5) accumulation. These glomerular structural changes were associated with a significant increase in urinary albumin excretion in 23-month-old mice (Fig. 6).

Aging was associated with increases in renal triglyceride and cholesterol content

There was an age-related increase in Oil Red O staining which started to become evident at 12 months of age (Fig. 7) and was indicative of neutral lipid accumulation. The increased Oil Red O accumulation was within

Table 2. Body weight, kidney weight, and kidney weight to body weight ratio for each age stratified by diet group

Treatment	3 months old		6 months old		12 months old		19 months old		23 months old	
	F	F/R	F	F/R	F	F/R	F	F/R	F	F/R
Body weight g	23.4 ± 0.34	26.1 ± 0.68	27.3 ± 0.77	29.0 ± 1.17	28.2 ± 0.68	28.7 ± 1.40	29.6 ± 0.18	31.9 ± 1.45	30.8 ± 1.68	30.7 ± 0.96
Kidney weight g	.141 ± .007	.127 ± .015	.150 ± .013	.163 ± .007	.181 ± .005	.167 ± .003	.189 ± .011	.189 ± .002	.172 ± .004	.172 ± .010
Kidney weight/body weight %	0.60 ± 0.02	0.49 ± 0.02	0.55 ± 0.04	0.56 ± 0.01	0.64 ± 0.01	0.59 ± 0.03	0.64 ± 0.04	0.57 ± 0.02	0.57 ± 0.05	0.56 ± 0.03

Values reported as mean ± SEM of six samples. F is fasting; F/R is fasting/refeeding.

Table 3. Serum data from each age group stratified by diet group

Treatment	3 months old		6 months old		12 months old		19 months old		23 months old	
	F	F/R	F	F/R	F	F/R	F	F/R	F	F/R
Glucose mg/dL	86.6 ± 1.28	160.4 ± 8.75	102.8 ± 11.0	138.6 ± 5.70	100.9 ± 9.30	133.8 ± 14.9	100.6 ± 12.5	129.6 ± 7.48	82.9 ± 6.51	128.6 ± 8.90
Cholesterol mg/dL	50.5 ± 2.31	71.5 ± 4.10	48.4 ± 7.26	57.4 ± 9.13	60.1 ± 5.09	68.8 ± 1.61	59.9 ± 12.3	66.2 ± 8.28	56.7 ± 2.75	63.2 ± 5.01
Triglycerides mg/dL	25.3 ± 3.79	37.4 ± 3.47	19.8 ± 7.01	24.1 ± 6.03	25.6 ± 5.34	26.2 ± 6.66	26.1 ± 4.65	25.1 ± 3.01	27.0 ± 3.78	27.0 ± 1.23
Nonesterified fatty acids mmol/L	0.83 ± 0.07	0.38 ± 0.01	0.75 ± 0.89	0.29 ± 0.03	0.87 ± 0.11	0.36 ± 0.05	0.58 ± 0.08	0.50 ± 0.04	0.71 ± 0.04	0.32 ± 0.01

Values reported as mean ± SEM of six samples. F is fasting; F/R is fasting/refeeding.

Table 4. Blood urea nitrogen (BUN) and serum creatinine

	3 months old	12 months old	23 months old
BUN mg/dL	31.73 ± 2.77	34.37 ± 2.08	33.70 ± 1.83
Serum creatinine mg/dL	0.25 ± 0.05	0.26 ± 0.04	0.29 ± 0.05

Values reported as mean ± SEM of six samples.

the glomerular mesangial cells. In addition, there was focal accumulation of staining in the proximal tubule cells (Fig. 7C and E), but this was not nearly as dramatic as that occurring in the glomerulus. The increase in Oil Red O accumulation corresponded to the increases in mesangial matrix protein accumulation.

Renal lipid composition studies indicated age-related increases in triglyceride (Fig. 8) and cholesterol (Fig. 9) content. These lipid compositional changes were seen both in 48-hour fasting mice (Figs. 8A and 9A) as well as in mice that were refed for 4 hours after the 48-hour fasting (Figs. 8B and 9B).

Aging was associated with increased SREBP-1 and SREBP-2 expression

Because SREBP-1 and SREBP-2 are the master transcriptional regulators of triglyceride and cholesterol synthesis, respectively, we next determined whether the age-related increases in renal triglyceride and cholesterol content were associated with increased expression of SREBP-1 and SREBP-2. Western blots of nuclear extracts from the kidney indicated that in both fasting (Figs. 10A and 11A) and refed (Figs. 10B and 11B) mice there were marked and significant increases in SREBP-1 (Fig. 10) and SREBP-2 (Fig. 11) protein abundance.

Aging was associated with increased expression of key enzymes that mediate fatty acid and cholesterol synthesis

The increase in nuclear SREBP-2 protein abundance was paralleled with significant increases in HMG-CoA reductase protein abundance (Fig. 12), a SREBP-2 regulated enzyme that plays a key role in cholesterol synthesis, and low-density lipoprotein (LDL) receptor mRNA level (Table 5), a SREBP-2 regulated receptor that plays a key role in cholesterol uptake. We also examined the age-dependent regulation of two key proteins that mediate cholesterol efflux. While there was no change in scavenger-receptor class B, type 1 (SR-B1) mRNA, there was an age-related increase in ATP-binding cassette A-1 (ABCA1) mRNA level (Table 5).

The increase in nuclear SREBP-1 protein abundance was paralleled with a significant increase in ATP citrate lyase activity (Fig. 13), a SREBP-1 regulated enzyme that plays an important role in fatty acid synthesis. The increase in ATP citrate lyase activity was evident at 12 months of age. After 12 months, the level of activity

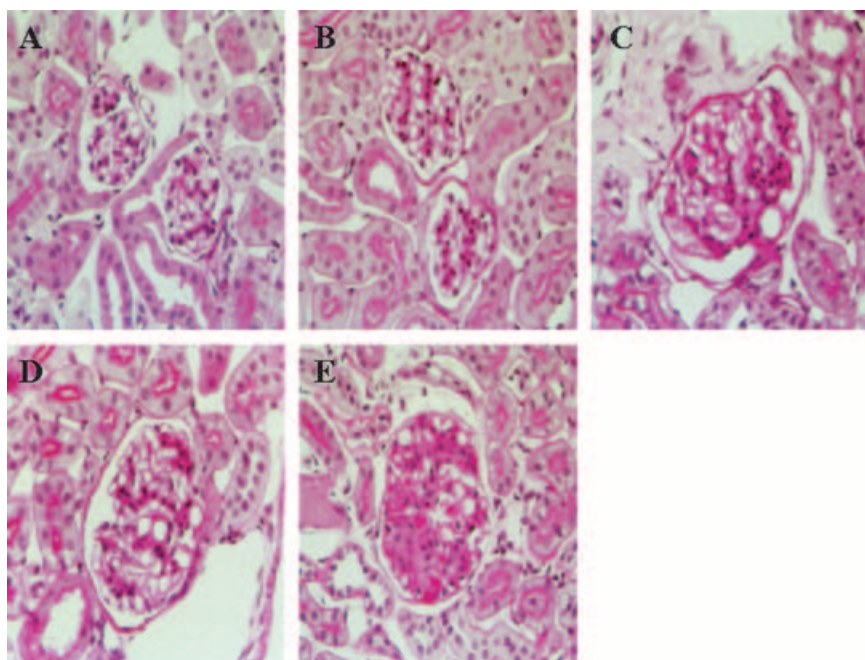


Fig. 1. Periodic acid-Schiff (PAS) staining of kidney cortex of 3-month-old (A), 6-month-old (B), 12-month-old (C), 19-month-old (D), and 23-month-old (E) mice. There was an age-associated increase in matrix accumulation, which was most evident after 12 months of age.

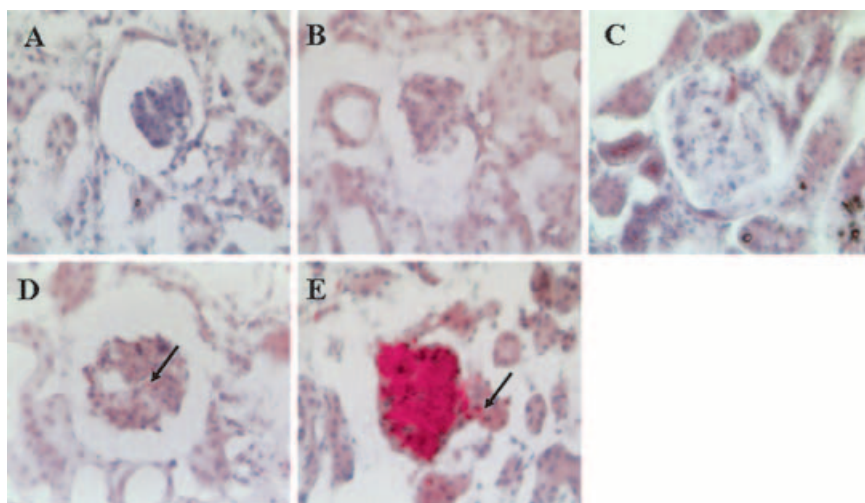


Fig. 7. Oil Red O staining for 3-month-old (A), 6-month-old (B), 12-month-old (C), 19-month-old (D), and 23-month-old (E) mice. The staining revealed increased accumulation of lipids (arrows) starting at 12 months, and becoming much more prominent in the 23-month-old mice.

for ATP citrates lyase plateaued, and seemed to mildly decrease again at 23 months of age. We also examined the age-dependent regulation of two key proteins that mediate fatty acid oxidation. There were no age-related significant changes in acetyl coenzyme A oxidase (ACO) or carnitine palmitoyl transferase 1 (CPT-1) mRNA levels (Table 5).

DISCUSSION

Our study was in agreement with earlier studies that have demonstrated increased incidence of age-related glomerulosclerosis in rats and mice [29, 30]. Histologic examination of *in vivo* perfused kidney sections demonstrated that the age-related increase in PAS staining was first evident at 12 months of age and then there was a

further age-related increase in PAS staining. Electron microscopy of parallel kidney sections indicated similar age-related glomerular changes and also age-related increase in glomerular basement thickness and podocyte width. Immunofluorescence microscopy of kidney sections also indicated an age-related increase in type IV collagen and fibronectin accumulation, which paralleled the finding with PAS staining.

The finding of age-related alteration in podocyte structure and increased foot process thickness in our study was in agreement with the reported literature. In the puromycin aminonucleoside-induced nephrosis model, podocytes showed effacement, pseudocysts, detachment from the glomerular basement membrane, and decreased anionic charge, resulting in proteinuria [31]. It has also been shown that in Milan normotensive rats, in

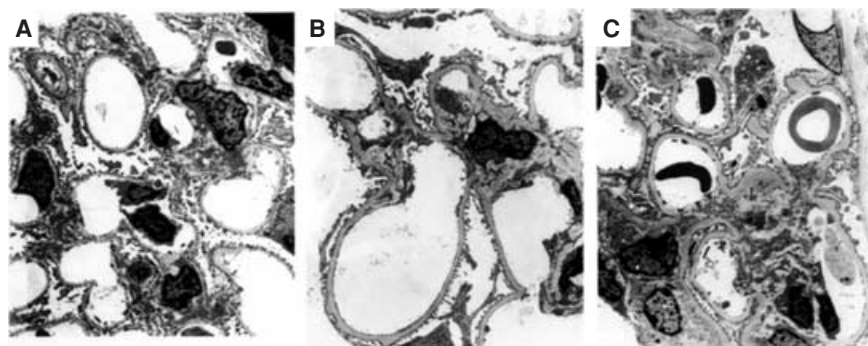


Fig. 2. Transmission electron microscopy of 3-month-old (A), 12-month-old (B), and 23-month-old (C) mice. There was an age-related increase in glomerular basement membrane thickness, as well as podocyte width. Increasing podocyte effacement was evident in the 12-month-old and 23-month-old mice.

which glomerulosclerosis and interstitial fibrosis developed with age in the absence of systemic hypertension, podocyte damage occurred as early as 3 months. The damage became more widespread with increasing age, culminating in a loss of podocytes, and sclerotic glomeruli [32]. In the fa/fa Zucker rat (a model of type 2 diabetes), the pathologic lesion of focal segmental glomerulosclerosis was associated with podocyte foot process effacement, pseudocyst formation, detachment from the basement membrane, and lipid droplet accumulation within the podocyte [33, 34]. In terms of human disease, podocyte abnormalities have been implicated in the nephropathy of type 1 diabetes mellitus [35], type 2 diabetes mellitus [36, 37], focal segmental glomerulosclerosis [38], minimal change disease [39], human immunodeficiency virus (HIV) nephropathy [40], IgA nephropathy [41], and lupus nephritis [38].

A novel finding of our study was the demonstration of age-related increases in Oil Red O staining, which indicated age-related increases in renal neutral lipid accumulation. Quantitative assays of lipid extracts from kidney indicated that the increases in Oil Red O staining corresponded to significant age-related increases in renal triglyceride and cholesterol content. These changes were seen both during fasting and postprandial conditions indicating that the changes could not be merely ascribed to alterations in the feeding cycle.

Because SREBP-1 and SREBP-2 are master regulators of fatty acid and cholesterol metabolism [42–44], respectively, we determined if there were age-related alterations in kidney SREBP-1 and SREBP-2 expression. We found that in nuclear extracts from the kidney there were significant age-related increases in the cleaved active nuclear form of SREBP-1 and SREBP-2.

We next examined the expression of key SREBP-1 target enzymes that mediate fatty acid synthesis. We found that there was an age-related increase in ATP citrate lyase activity, a key enzyme that mediates increased fatty acid synthesis and results in increased triglyceride synthesis. Because triglyceride accumulation can also be mediated by decreased fatty acid oxidation, we also measured the mRNA levels of two key enzymes that mediate fatty acid

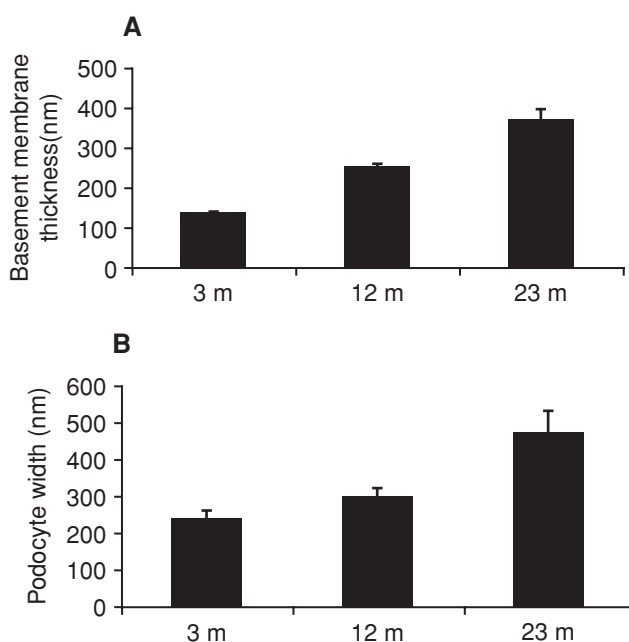


Fig. 3. Basement membrane thickness (A) and podocyte width (B) of 3-month-old, 12-month-old, and 23-month-old mice. Both basement membrane thickness and podocyte width increased in an age-dependent manner ($P < 0.0001$ for trend of increase in both basement membrane thickening and podocyte width thickening). Comparisons of individual ages revealed significant differences between 3 months vs. 12 months and 23 months, and 12 months vs. 23 months for basement membrane thickness, and between 3 months vs. 23 months and 12 months vs. 23 months for podocyte width.

oxidation. We found no age-related changes in ACO or CPT-1 mRNA levels, indicating no age-related decreases in fatty acid oxidation. These studies therefore suggested that increased age-related fatty acid synthesis rather than decreased fatty acid oxidation mediated the age-related increase in triglyceride accumulation in the kidney. As reported recently, in models of renal tubular injury renal triglyceride accumulation can also be mediated by increased fatty acid uptake via the fatty acid binding protein or decreased degradation via decreased lipase activity [45]. These pathways need to be elucidated in future studies.

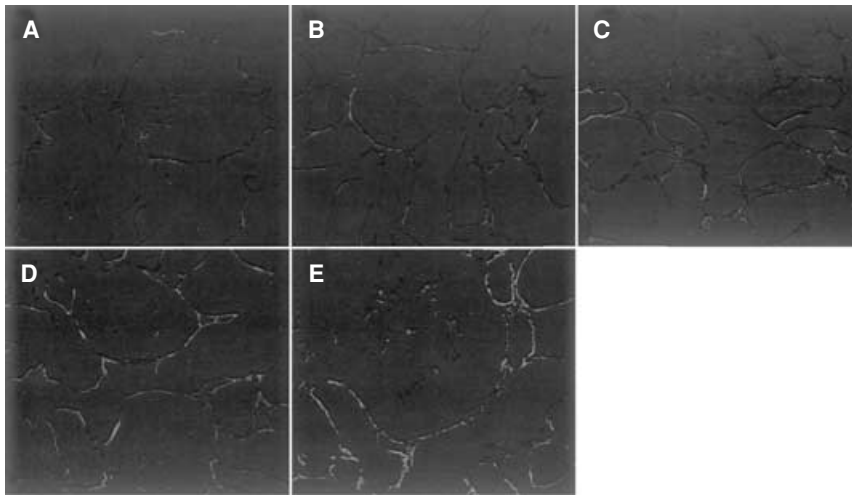


Fig. 4. Immunofluorescence staining of renal cortex for type IV collagen in 3-month-old (A), 6-month-old (B), 12-month-old (C), 19-month-old (D), and 23-month-old (E) mice. There was increased type IV collagen deposition with age.

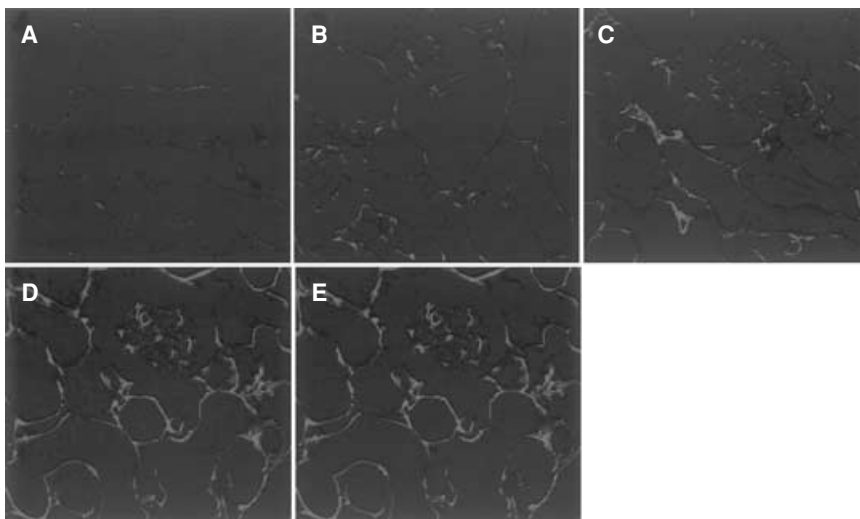


Fig. 5. Immunofluorescence staining of renal cortex for fibronectin in 3-month-old (A), 6-month-old (B), 12-month-old (C), 19-month-old (D), and 23-month-old (E) mice. There was increased glomerular fibronectin deposition with age.

We also examined the expression of key SREBP-2 target enzymes that mediate cholesterol synthesis and/or cholesterol uptake. We found that there was an age-related increase in HMG-CoA reductase mRNA and protein abundance, a key enzyme that mediates increased cholesterol synthesis, and mRNA level of LDL receptor, which mediates cholesterol uptake. Because cholesterol accumulation can also be mediated by decreased cholesterol efflux [46, 47], we also measured the mRNA levels of two key proteins that mediate cholesterol efflux. We found no age-related decrease in SR-B1 mRNA level but a significant age-related increase in ABCA1 mRNA level. These studies therefore suggested that increased age-related cholesterol synthesis and uptake rather than decreased cholesterol efflux mediated the age-related increase in cholesterol accumulation in the kidney.

The regulation of SREBP expression in the kidney has not been well described. However, studies in other organ systems have generated a number of possible candidates involved in SREBP regulation. Previous stud-

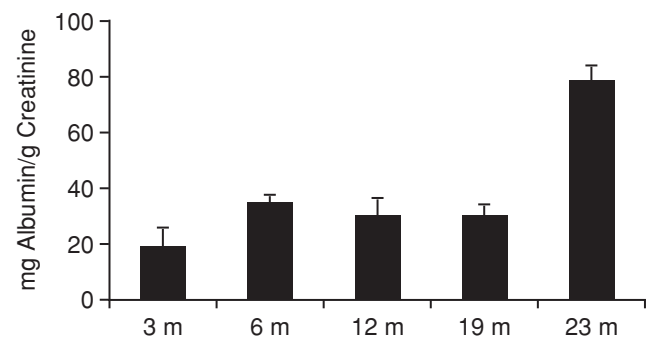


Fig. 6. Urinary protein excretion for the different ages of mice, expressed as urinary albumin to creatinine ratio. There was a significant age-related increase in urinary protein excretion.

ies in the liver have demonstrated that SREBP-1c and SREBP-2 appear to be subjected to transcriptional regulation, whereas SREBP-1a appears to be constitutively expressed at low levels [48]. Excess insulin stimulates

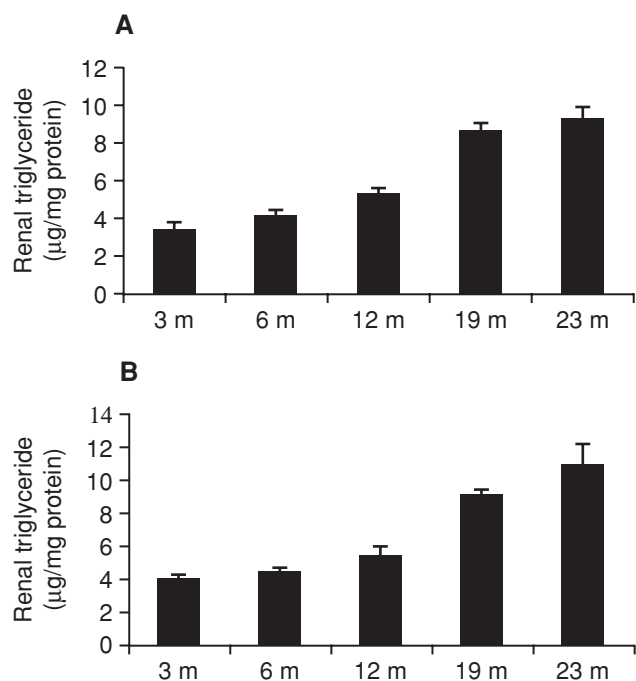


Fig. 8. Renal triglyceride content of mice from different age groups. (A) Fasting mice. The mice showed a statistically significant increase in renal triglyceride content as a function of aging (P for trend <0.01). Comparisons of individual ages revealed significant differences between 3 months vs. 12, 19, and 23 months, 6 months vs. 19 and 23 months, and 12 months vs. 19 and 23 months. (B) Refed mice. The mice showed a statistically significant increase in renal triglyceride content as a function of aging (P for trend <0.01). Comparisons of individual ages revealed significant differences between 3 months vs. 19 and 23 months, 6 months vs. 19 and 23 months, and 12 months vs. 19 and 23 months.

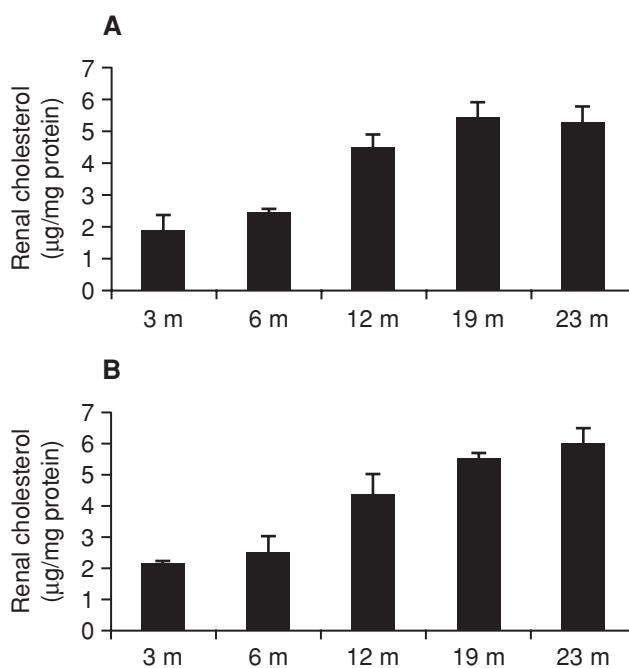


Fig. 9. Renal cholesterol content of mice from different age groups. (A) Fasting mice. The mice showed a statistically significant increase in renal cholesterol content as a function of aging (P for trend <0.01). Comparisons of individual ages revealed significant differences between 3 months vs. 12, 19 and 23 months, and 6 months vs. 12, 19 and 23 months. (B) Refed mice. The mice showed a statistically significant increase in renal cholesterol content as a function of aging (P for trend <0.01). Comparisons of individual ages revealed significant differences between 3 months vs. 12, 19, and 23 months, 6 months vs. 12, 19, and 23 months, and 12 months vs. 23 months.

fatty acid synthesis in the liver in periods of carbohydrate excess. Studies in hepatocyte cell culture [49, 50] showed that treatment with insulin increased SREBP-1c mRNA. Fasting/refeeding studies showed that the amount of SREBP-1c in liver depended on the state of feeding. Fasting decreased whereas refeeding increased liver SREBP-1c, respectively. This paralleled the changes in insulin secretion [44]. Studies have also suggested that increased insulin levels or changes in nutritional status from fasting to fed also upregulated SREBP-1c mRNA levels in other organs, such as skeletal muscle [51, 52] and adipose tissue [53, 54]. In addition, streptozotocin treatment (which abolishes insulin secretion) led to a fall in SREBP-1c mRNA in the liver, which was reversed upon exogenous treatment with insulin [50]. Interestingly, our laboratory has found the opposite response in the kidneys of streptozotocin-treated mice, suggesting differential or tissue-specific regulation of SREBP-1 in these two organs [15].

Leptin deficiency has recently been implicated as a potential mediator of increased SREBP-1c in adipose tissue [55]. In leptin-deficient ob/ob mice, an increase of liver SREBP-1c has been reported [56], although these ani-

mals tended to be hyperinsulinemic as well, making it difficult to ascribe the increase in SREBP solely to the leptin resistance per se.

Recently, tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 β have been demonstrated to increase nuclear SREBP-1 protein in mesangial cell culture by increasing SREBP cleavage activating protein (SCAP) mRNA expression [57]. Other potential mediators of increased SREBPs include IL-6 [58], and growth factors such as epidermal growth factor (EGF) [59].

We propose that the age-related increases in renal SREBP-1 and SREBP-2 expression and the resultant increases in renal triglyceride and cholesterol content mediate the age-related glomerulosclerosis. This proposal is supported by several lines of evidence.

In SREBP-1a transgenic mice that overexpressed the SREBP-1a transgene in the kidney, in the absence of any increases in serum glucose, triglyceride, or cholesterol, there was a significant increase in renal lipid deposits and an increase in renal triglyceride content, which was associated with glomerulosclerosis and proteinuria, resembling the renal pathology seen in the diabetic and aging kidney [15].

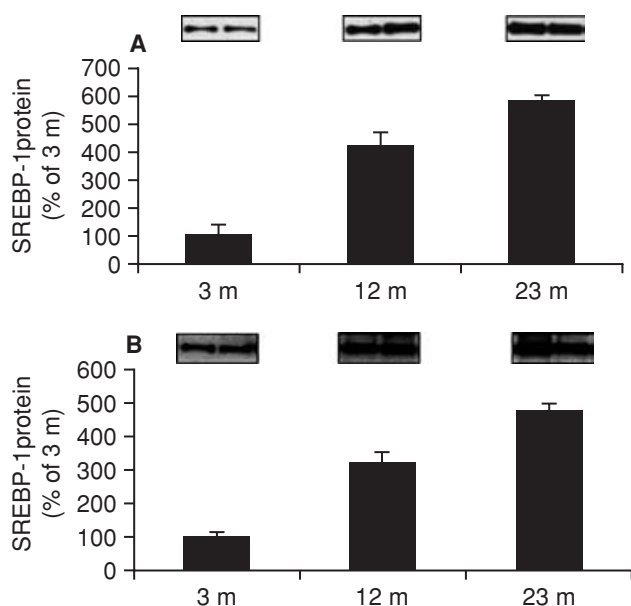


Fig. 10. Nuclear sterol regulatory element-binding proteins (SREBP-1) protein levels in kidney tissue for each age group. (A) Fasting mice. There was an age-related increase in the amount of renal nuclear SREBP-1 protein (P for trend < 0.01). Comparisons of individual ages revealed significant differences between 3 months vs. 12 and 23 months, and 12 months vs. 23 months. (B) Refed mice. There was an age-related increase in the amount of renal nuclear SREBP-1 protein (P for trend < 0.01). Comparisons of individual ages showed significant differences between 3 months vs. 12 and 23 months, and 12 months vs. 23 months.

In addition, renal glomerular and tubular disease occurred in a number of animal and human conditions with primary alteration in lipid metabolism, including in the apolipoprotein E (ApoE) knockout mice [60], in human subjects with lecithin cholesterol acyltransferase (LCAT) deficiency [61], in ABCA1 knockout mice, a murine model of Tangiers disease and familial high-density lipoprotein (HDL) deficiency, with defects in ABCA1 and HDL mediated reverse cholesterol transport [62], in Fabry disease, an X-linked recessive inborn error of glycosphingolipid catabolism resulting from deficient activity of the lysosomal hydrolase α -galactosidase A [63], and in congenital and acquired lipodystrophy [64].

There is also increasing evidence that inhibition of cholesterol synthesis by HMG-CoA reductase inhibitors (statins) and triglyceride synthesis by peroxisome proliferators-activated receptor- α (PPAR- α) agonists (fibrates) protected against diabetic and nondiabetic renal disease [65, 66]. A recent meta-analysis of several small scale interventional studies in diabetic and nondiabetic human subjects with glomerulosclerosis and proteinuria in fact indicated that long-term treatment with statins and/or fibrates significantly prevent the decline in GFR [67].

The mechanisms by which lipids mediate glomerulosclerosis are not completely established. In the SREBP-1a transgenic mouse we have found increased renal

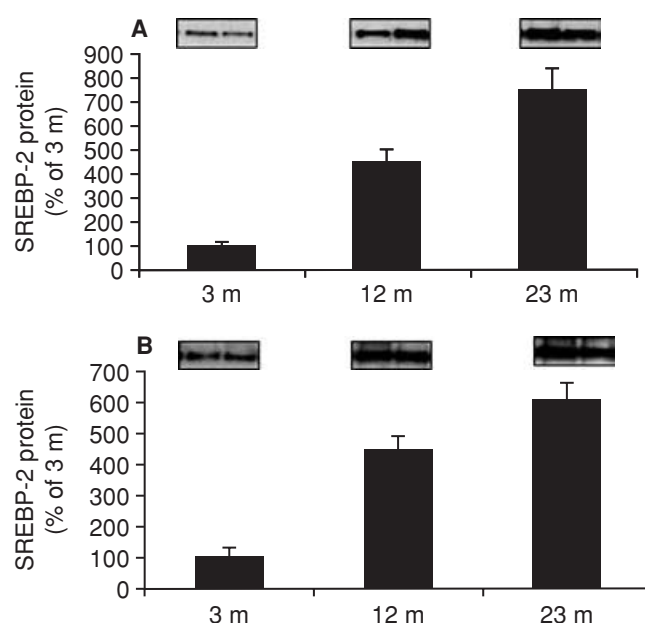


Fig. 11. Nuclear sterol regulatory element-binding proteins (SREBP-2) protein levels in kidney tissue for each age group. (A) Fasting mice. There was an age-related increase in the amount of renal nuclear SREBP-2 protein (P for trend < 0.01). Comparisons of individual ages showed significant differences between 3 months vs. 12 and 23 months, and 12 months vs. 23 months. (B) Refed mice. There was an age-related increase in the amount of renal nuclear SREBP-2 protein (P for trend < 0.01). Comparisons of individual ages showed significant differences between 3 months vs. 12 and 23 months, and 12 months vs. 23 months.

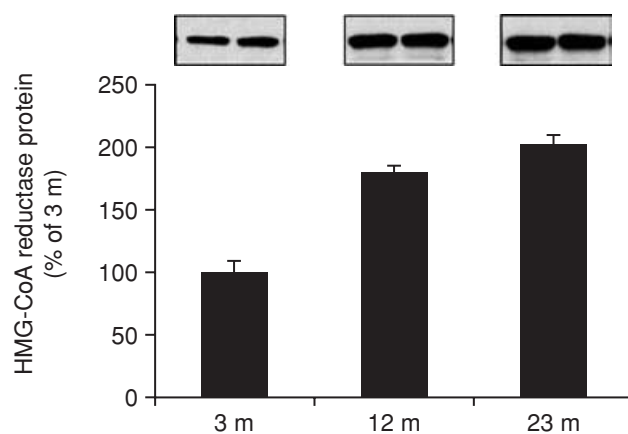


Fig. 12. 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase protein abundance for each 3, 12, and 23-month old mice. There was an age-related increase in HMG-CoA reductase protein levels (P for trend < 0.01). A comparison of individual ages showed significant differences between 3 months vs. 12 and 23 months.

expression of TGF- β and VEGF resulting in increased expression of type IV collagen and fibronectin, glomerulosclerosis, and proteinuria [15]. In addition, cell culture studies have shown that in mesangial cells LDL or VLDL induced up-regulation of expression of TGF- β [68], plasminogen activator inhibitor-1 (PAI-1) [69], and monocyte chemoattractant protein-1 (MCP-1) [70], and

Table 5. mRNA level of enzymes involved in lipid metabolism in the kidneys

	3 months old	12 months old	23 months old
Fatty acid synthesis			
SREBP-1c	17.54 ± 7.53	34.75 ± 8.14	47.07 ± 11.01 ^a
Fatty acid oxidation			
ACO	149.37 ± 18.19	137.73 ± 25.04	112.17 ± 20.12
CPT-1	8.32 ± 3.29	10.43 ± 2.28	9.21 ± 2.04
Cholesterol synthesis			
HMG-CoA reductase	37.04 ± 6.67	45.65 ± 11.45	55.20 ± 5.13 ^a
Cholesterol efflux			
ABCA1	1.66 ± 0.21	1.89 ± 0.32	3.27 ± 0.63 ^a
SR-B1	3.45 ± 0.80	3.18 ± 0.64	3.12 ± 0.19
Cholesterol uptake			
LDL receptor	76.52 ± 30.80	76.70 ± 24.31	198.84 ± 47.26 ^{a,b}

Abbreviations are: ACO, acetyl coenzyme A oxidase; CPT-1, carnitine palmitoyltransferase I; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ABCA1, ATP-binding cassette A-1; SR-B1, scavenger-receptor class B, type 1; LDL receptor, low-density lipoprotein receptor.

Values reported as mean ± SEM (arbitrary unit) of six samples.

^a*P* < 0.05 vs. 3 months; ^b*P* < 0.05 vs. 12 months.

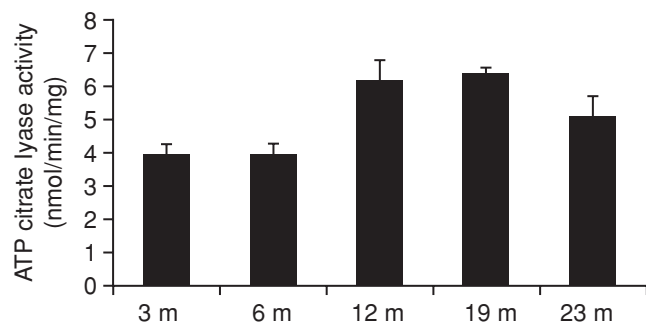


Fig. 13. Adenosine triphosphate (ATP) citrate lyase activity for each age group of mice. There was an increase in ATP citrate activity up to 19 months, and then a decrease was seen at 23 months. The increasing trend was significant (*P* = 0.0022). Comparisons of individual ages showed significant differences between 3 months vs. 12 and 19 months, and 6 months vs. 12 and 19 months.

accumulation of extracellular matrix proteins [71], indicating a direct role for lipids in activating the mediators of glomerulosclerosis.

Of course as elegantly shown by Johnson, Stahl and Zager [45], Zager et al [46], and Johnson, Yabu, and Zager [47], the possibility also exists that age-related renal injury per se can activate the lipid metabolism pathways rather than the alterations in renal lipid metabolism mediating the age-related renal disease. However, we did provide evidence that there was age-related intricate regulation of renal lipid metabolism that resulted in net renal triglyceride and cholesterol accumulation, and that increased activity of SREBP per se can induce glomerulosclerosis and proteinuria [15].

CONCLUSION

Our results indicated that there was an age-related increase in renal expression of SREBP-1 and SREBP-2

with resultant increases in lipid synthesis and triglyceride and cholesterol accumulation in the kidney. Because we have previously shown that increased expression of SREBPs in the kidney per se resulted in glomerulosclerosis and proteinuria, our data suggested that increased SREBPs expression resulting in increased renal lipid accumulation plays an important role in age-related nephropathy.

ACKNOWLEDGMENTS

These studies were supported by grants to Moshe Levi (NIH 7R03 AG20361-02, NIH 5R01 DK062209-02, JDRF 1-2003-108, and AHA 0350491Z) and Scott Liebman (NIH NRSA 1F32 DK065407-01). The authors thank Dr. Nabil Halaihel and Dr. William L. Gitomer for their assistance with these studies.

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